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A Highly Specific Mechanism of Histone H3-K4 Recognition by Histone Demethylase LSD1*

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Human lysine-specific demethylase (LSD1) is a chromatin-modifying enzyme that specifically removes methyl groups from mono- and dimethylated Lys[4] of histone H3 (H3-K4). We used a combination of *in vivo* and *in vitro* experiments to characterize the substrate specificity and recognition by LSD1. Biochemical assays on histone peptides show that essentially all epigenetic modifications on the 21 N-terminal amino acids of histone H3 cause a significant reduction in enzymatic activity. Replacement of Lys[4] with Arg greatly enhances binding affinity, and a histone peptide incorporating this mutation has a strong inhibitory power. Conversely, a peptide bearing a trimethylated Lys[4] is only a weak inhibitor of the enzyme. Rapid kinetics measurements evidence that the enzyme is efficiently reoxidized by molecular oxygen with a second-order rate constant of 9.6×10^3 M^{-1} s^{-1}, and that the presence of the reaction product does not greatly influence the rate of flavin reoxidation. *In vivo* experiments provide a correlation between the *in vitro* inhibitory properties of the tested peptides and their ability of affecting endogenous LSD1 activity. Our results show that epigenetic modifications on histone H3 need to be removed before Lys[4] demethylation can efficiently occur. The complex formed by LSD1 with histone deacetylases 1/2 may function as a "double-blade razor" that first eliminates the acetyl groups from acetylated Lys residues and then removes the methyl group from Lys[4]. We suggest that after H3-K4 demethylation, LSD1 recruits the forthcoming chromatin remodelers leading to the introduction of gene repression marks.

Post-translational modifications on histone proteins are fundamental epigenetic marks that control chromatin state and gene expression (1, 2), and unraveling epigenetic mechanisms is a new area of biomedical research (3). Histone lysine demethylases participate in the regulation of chromatin functional states by removing methyl groups from lysine residues on histone N-terminal tails. At present two different subclasses of histone lysine demethylases have been identified: the FAD-dependent histone demethylases (4) (Fig. 1) and the JmjC domain containing histone demethylases, representing iron-dependent dioxygenases that use 2-oxoglutarate for histone lysine demethylation via hydroxylation (5). LSD1 was the first discovered histone demethylase and so far, the only one known to require a flavin cofactor (4, 6). This enzyme specifically acts on mono- and dimethylated Lys[4] of histone H3 (H3-K4), and its activity induces gene repression because H3-K4 methylation is generally associated with activation (7). The enzyme is part of several multiprotein corepressors including CoREST, CtBP, and a subset of histone deacetylases 1/2 complexes (8–12). The LSD1 catalyzed reaction starts with the oxidation of the H3-K4 *N*-methyl group carried out by the FAD cofactor (Fig. 1) . The resulting imine intermediate is then hydrolyzed generating the demethylated histone and formaldehyde.

We used a combination of *in vivo* and *in vitro* experiments in an effort to characterize LSD1 substrate specificity and mechanism of H3-K4 recognition. We provide evidence that oxygen can be the physiological electron acceptor, and that the enzyme is likely to function through a ternary complex mechanism. In addition, our data show that LSD1 can efficiently act only after removal of the other epigenetic modifications present on the same histone H3 N-terminal tail.

EXPERIMENTAL PROCEDURES

Protein Preparation and Steady-state Kinetics Measurements—All chemicals were purchased from Sigma unless specified. Human LSD1 lacking the N-terminal 157 amino acids was expressed and purified as described (6, 13). Enzymatic activities were measured under aerobic conditions by using a peroxidase-coupled assay (13) on a Cary 100 UV/visible spectrophotometer. Peptides were purchased from Thermo Electron Corporation. Their purity was greater than 90% as checked by analytical high pressure liquid chromatography and mass spectrometry. LSD1 inhibitors were tested by using the peroxidase-coupled assay in the presence of varied concentrations (2–100 μM) of monomethylated H3-K4 peptide and of the inhibitor under analysis (global range 1–300 μM, depending on the inhibitor

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[4] The abbreviations used are: LSD1, lysine-specific demethylase; H3-K4, Lys[4] of histone 3.

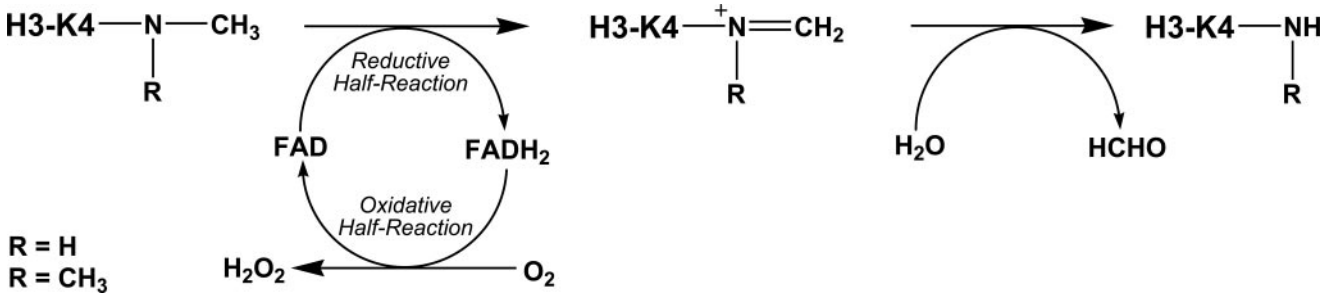

FIGURE 1. **Histone H3 Lys[4] demethylation reaction catalyzed by LSD1.** Mono- or dimethylated H3-K4 is oxidized by the FAD cofactor (reductive half-reaction) to an imine intermediate that is hydrolyzed to yield the demethylated histone tail and formaldehyde. In the oxidative half-reaction, the FAD is reoxidized by an electron acceptor such as molecular oxygen.

strength). Initial velocity values were fitted to equations describing competitive, uncompetitive, and noncompetitive inhibition patterns using Grafit (Erithacus Software) to obtain the values of apparent k_{cat} and K_m along with their associated errors. Propagation of statistical error value was carried out as described (14).

Stopped Flow Kinetics of the Oxidative Half-reaction—Stopped flow kinetic experiments were performed using a SX17MV stopped-flow instrument equipped with a diode array detector (Applied Photophysics, Leatherhead, UK). For these experiments, the enzyme was artificially reduced by preparing a solution of 10 μM enzyme in 50 mM HEPES, pH 7.5, also containing benzyl viologen (1.0 μM), and xanthine (400 μM) in a total volume of 1.2 ml. After flushing this enzyme solution with nitrogen, enzyme reduction was initiated by adding 0.2–0.5 nM xanthine oxidase resulting in full reduction of LSD1 within 30 min. The reduced LSD1 was mixed with buffer (50 mM HEPES, pH 7.5) with varying oxygen concentrations. Upon mixing in the stopped-flow instrument, spectral scans were collected either in 4 or 8 ms intervals. The same experiment was repeated in the presence of 100 μM unmodified 21-amino acid histone H3 peptide. The spectral data were analyzed using the Pro-K software package (Applied Photophysics).

Cell Line Maintenance and Peptide Transduction—HEK293 cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamax (Invitrogen), 10% fetal bovine serum, 1% penicillin/streptomycin (Celbio) and grown in humidified incubators at 37 °C and 5% CO_2. HEK293 cells were transduced with selected peptides complexed with the Chariot reagent (Active Motif, Carlsbad, CA). In brief, 1×10^6 HEK293 cells/well were seeded in 35-mm culture plates and incubated until they were at 50% confluence. For each transduction reaction, 6 μl of a 1:10 diluted Chariot solution was added to 100 μl of sterile water, and 500 ng of peptide was diluted in 100 μl of phosphate-buffered saline. Peptide was added to Chariot dilution and then incubated for 30 min at room temperature. Cells were overlaid with the Chariot-peptide complex, and 400 μl of serum-free medium was added to the overlay achieving the final transfection volume of 600 μl. One hour after transfection, 1 ml of complete growth medium was added to each well without removing the Chariot-peptide complex. Cells were incubated again at 37 °C in a humidified atmosphere containing 5% CO_2 for 1.5 h. In control experiments, cells were treated with the Chariot reagent without peptide.

Isolation of Total RNA and Real-time Quantitative Reverse Transcription-PCR Analysis—Total RNA was isolated from treated or control cells using the RNA Wiz™ reagent (Ambion) according to the manufacturer's protocol. Real-time PCRs were performed on the iQ5 real-time PCR detection system (Bio-Rad) using the iScrip™ one-step reverse transcription-PCR kit with SYBR® Green. The specificity of the amplifications was assessed by electrophoretic separation of the amplified products and melting curve analysis. The expression of the investigated genes was quantified after normalization with β2-microglobulin gene (*B2M*) expression. Primers used for amplification are the following: *B2M* forward TGCTGTCTCCATGTTTGATGTATCT, *B2M* reverse TCTCTGCTCCCCACCTCTAAGT; β-actin forward GGAGAAAATCTGGCACCACACC, β-actin reverse GCTGGGGTGTTGAAGGTCTCAA; tubulin forward CTCTGTTCGCTCAGGTCCTTTTG, tubulin reverse GCCTCCTTCCGTACCACATCC; *SCG10* forward GAAGAAAGACCTGTCCCTG, *SCG10* reverse GTTTCAGCACCTGGGCCTCC; *SCN1A* forward CATCGCCTGTTGGACAGCTT, *SCN1A* reverse AGTGGTTGTTCCATTGTCATCAG.

Protein Isolation and Western Blotting—Total proteins were extracted using radioimmune precipitation assay buffer, and Western blot analysis was carried out using standard techniques. The antibodies used were anti-LSD1 ab17721 (Abcam) and anti-α/β-tubulin (Cell Signaling).

RESULTS

Influence of Other Epigenetic Marks on Substrate Recognition and Enzymatic Activity—Substrate recognition by LSD1 is not confined to residues neighboring Lys[4] but it is achieved through a network of specific interactions with the 21 N-terminal residues of histone H3 (4, 13). The histone H3 N-terminal tail has many potential sites of epigenetic post-translational modifications, and it was shown that the locus Lys[9]-Ser[10] is particularly important in affecting LSD1 activity (13). To investigate how different epigenetic marks affect the LSD1 demethylation process, we tested several 21-amino acid peptides that in addition to being monomethylated on Lys[4] contain other epigenetic covalent modifications (Fig. 2, *a* and *b*). For each peptide, we measured the enzymatic activity with a peroxidase-coupled assay (Ref. 13 and Table 1), and the resulting steady-state kinetic

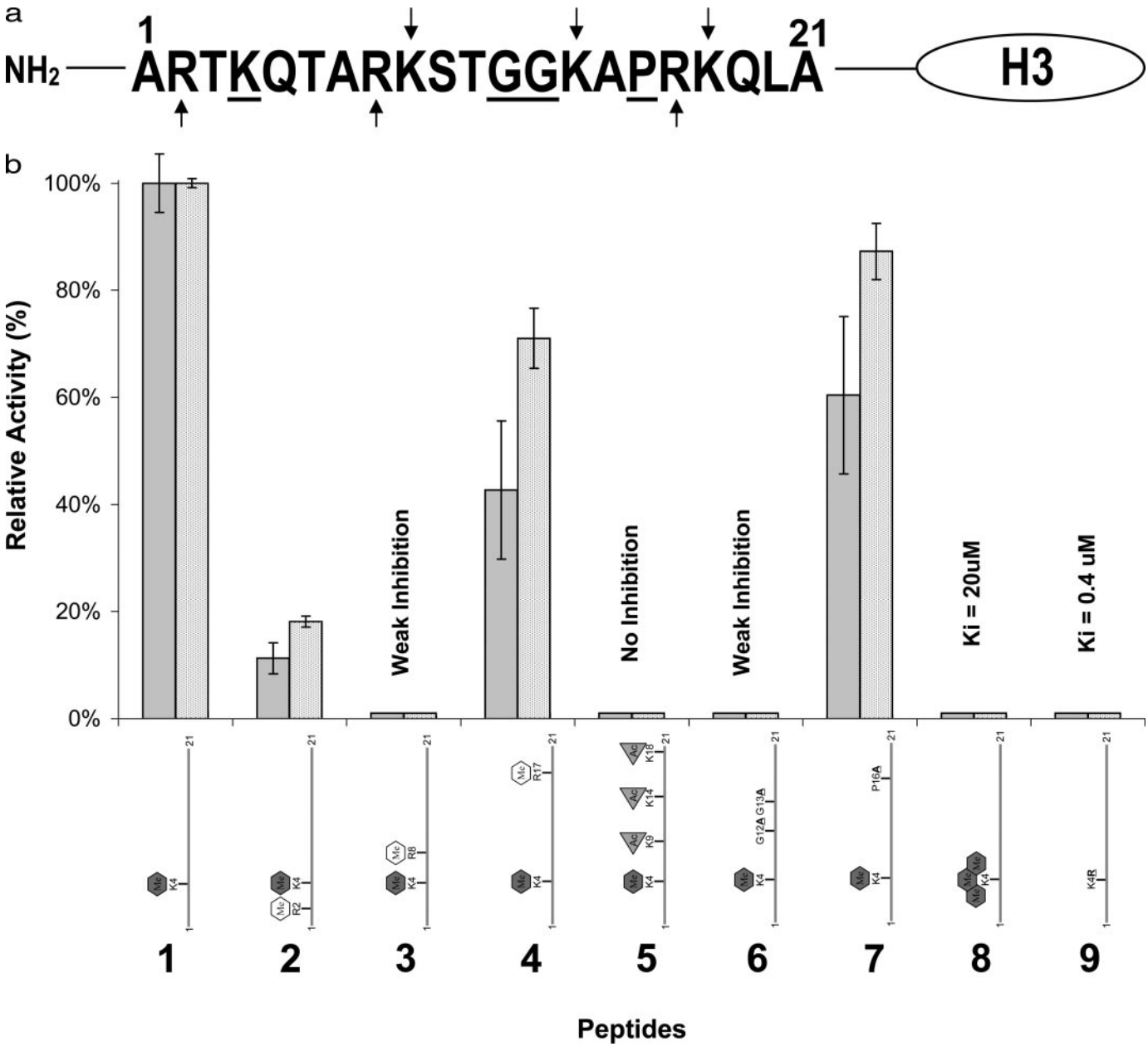

FIGURE 2. *a*, sequence of the first 21-amino acids of histone H3 N-terminal tail. This sequence is recognized by LSD1 and is necessary for efficient catalysis. *Arrows* indicate the sites of modifications introduced into the 21-amino acid peptides characterized in this study. Residues that have been investigated by mutagenesis are *underlined*. *b*, alteration (percentage) in LSD1 activity for the H3 peptides used in this study. *Solid bars* indicate the k_{cat}/K_m ratio, whereas the *dotted bars* refer to the k_{cat} values. For the peptides that are not LSD1 substrates, the inhibition effect was evaluated. K_i values were determined for those peptides that showed a significant decrease in H3-K4 demethylase activity. *Weak inhibition* indicates that an inhibitory effect was barely detectable ($K_i > 100$ μM). *Error bars* are shown, and propagation of statistical error value was carried out as described (14).

parameters were evaluated relatively to those obtained with the 21-amino acid monomethylated substrate (peptide 1).

We first tested the effect of arginine methylation, an epigenetic mark that has been recently shown to be dynamically modulated (15, 16). Monomethylation of Arg^2 (peptide 2) decreased LSD1 activity by more than 80%, whereas a methyl group on Arg^8 (peptide 3) made the peptide totally unable to function as a substrate. Arg^{17} monomethylation (peptide 4) had a lower effect mostly due to a decrease in binding affinity. We also found that peptide 3 hardly inhibits LSD1 ($K_i > 100$ μM) implying that methylation of Arg^8 completely prevents binding to the enzyme.

Next, we investigated the effect of lysine hyperacetylation, a post-translational modification of histone H3 that is associated with gene activation (3). For this purpose, a monomethylated H3-K4 peptide acetylated at Lys^9, Lys^{14}, and Lys^{18} (peptide 5) was tested. This peptide did not exhibit any inhibitory or catalytic activity. This observation is in agreement with data reported by Shi *et al.* (11) showing that hyperacetylated nucleosomes are less susceptible to CoREST/LSD1-mediated demethylation. Lysine acetylation seems to have an additive effect because acetylation at Lys^9 causes a 6-fold reduction in activity (13), whereas acetylation of all three Lys residues present in the 21-amino acid peptide leads to complete inactivation. Taken

TABLE 1
Kinetic parameters for the H3 peptides (21 amino acids) tested in peroxidase-coupled assay with LSD1

Peptide	k_{cat} [a]	K_m [a]	k_{cat}/K_m [a]	K_i [b]
	min^{-1}	μM	$\mu M^{-1}\ min^{-1}$	μM
1. Monomethyl Lys^4	3.4 ± 0.1	3.4 ± 0.2	1.0 ± 0.1	
2. Monomethyl Arg^2, monomethyl Lys^4	0.63 ± 0.03	5.5 ± 1.4	0.11 ± 0.02	
3. Monomethyl Lys^4, monomethyl Arg^8	No activity	No activity	No activity	Weak
4. Monomethyl Lys^4, monomethyl Arg^{17}	2.5 ± 0.2	5.7 ± 1.7	0.43 ± 0.13	
5. Monomethyl Lys^4, acetyl Lys^9, Lys^{14}, Lys^{18}	No activity	No activity	No activity	No inhibition
6. Monomethyl Lys^4, mutated G12A, G13A	No activity	No activity	No activity	Weak
7. Monomethyl Lys^4, mutated P16A	3.0 ± 0.2	4.9 ± 1.2	0.61 ± 0.15	
8. Trimethyl Lys^4	No activity	No activity	No activity	19.5 ± 3.2
9. Mutated K4R	No activity	No activity	No activity	0.41 ± 0.05
10. Unmodified peptide (residues 1–21)[c]	No activity	No activity	No activity	1.8 ± 0.6
11. Shorter unmodified peptide (residues 5–21)[c]	No activity	No activity	No activity	87 ± 29

[a] Apparent steady-state kinetic parameters determined as described under "Experimental Procedures." No activity, the activity is absent or barely detectable.
[b] Inhibition assays were performed for all the peptides that showed no activity in the peroxidase-coupled assay. The inhibition constant was determined in an assay with peptide 1 as substrate. Values were determined by using competitive inhibition algorithm from the Grafit software package (Erithacus Software). Weak indicates that only a weak inhibitory effect was detectable ($K_i > 100\ \mu M$). Propagation of statistical error value was carried out as described (14).
[c] Data taken from Forneris *et al.* (13).

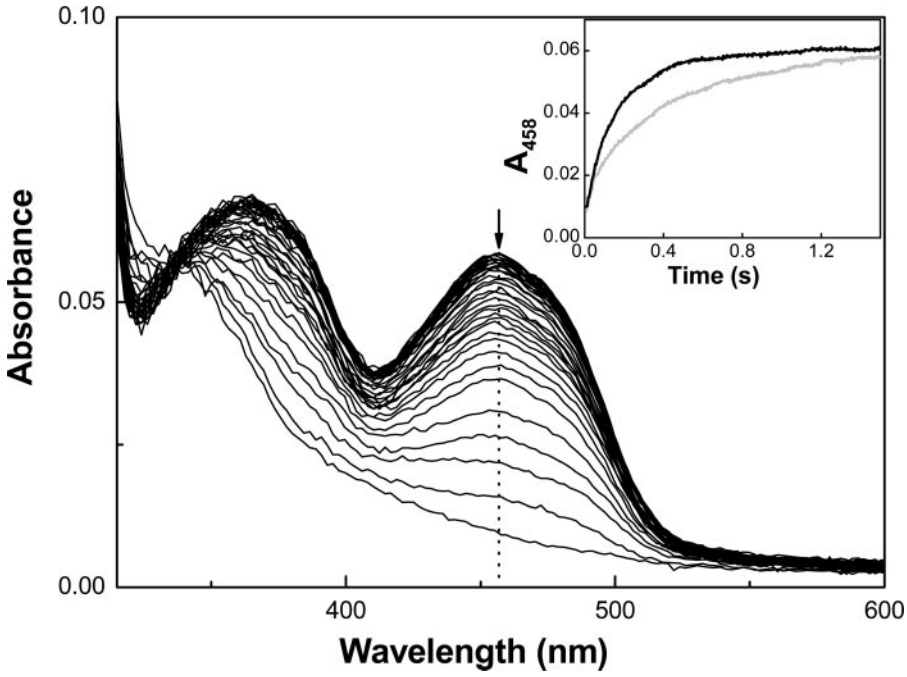

FIGURE 3. **Spectral changes observed upon mixing 10 μM fully reduced LSD1 with buffer containing 1.14 mM O_2.** Spectra were collected every 4 ms; only spectra obtained with 20-ms intervals are shown. After deconvolution, the spectral data could be fitted with a single exponential decay function yielding a rate of 5.5 s^{-1}. The *inset* shows the absorbency at 458 nm as a function of time (*black line*). The *gray line* in the *inset* refers to an analogous experiment performed in the presence of 100 μM inhibitor (peptide 10).

together, these experiments demonstrate that Lys^4 demethylation by LSD1 can be drastically reduced by other epigenetic marks present on the same histone H3 tail.

The Essential Role of Gly^{12}-Gly^{13} of Histone H3—To investigate whether LSD1 activity is influenced by specific stereochemical and conformational properties of the histone tail, we inserted point mutations in the H3-K4 monomethylated peptide sequence (Fig. 2, *a* and *b*). We designed two peptides, one with both Gly^{12} and Gly^{13} mutated to alanine (peptide 6) and another with Pro^{16} replaced by alanine (peptide 7). We chose glycine and proline as sites for mutagenesis because these amino acids can either restrain the peptide conformation (Pro^{16}) or provide the peptide with the conformational flexibility and/or adaptability required for binding (Gly^{12}-Gly^{13}). Peptide 7 exhibited normal catalytic properties suggesting that the conformational constraint imposed by Pro^{16} ring was not critical for productive binding to LSD1. Conversely, peptide 6 did not function either as a substrate or as an effective inhibitor indicating that the double mutation at Gly^{12}-Gly^{13} made the peptide unable to bind to the protein.

Specificity at the H3-K4 Site—The three-dimensional structure determination of LSD1 in the substrate-free state reveals a deep negatively charged pocket in proximity of the FAD cofactor and a shallow groove that might form the substrate-binding site (17, 18). We explored the specific properties of the Lys^4 binding site by using a trimethylated H3-K4 peptide and a peptide bearing an arginine at position 4. In the former, the charge on Lys^4 was embedded by the three methyl groups, whereas in the latter the positive charge was delocalized on the Arg guanidium group. As expected, we found that the trimethylated H3-K4 peptide (peptide 8) is not a substrate for LSD1, in agreement with the chemical nature of the flavin-dependent amine oxidation reaction that requires a free lone pair of electrons on the substrate nitrogen atom (19). Furthermore, peptide 8 was found to competitively inhibit LSD1 (K_i, 20 μM, Fig. 2*b*) but to a lower extent compared with the unmodified product (K_i, 2 μM; Table 1) (13). These data show that the enzyme senses the presence of the third methyl group so that LSD1 is both catalytically inactive against trimethylated Lys^4 and unable to bind it with high affinity. In this respect, it is worthwhile to note that LSD1 is much more efficient in discriminating among H3-K4 methylation states compared with other chromatin remodeling enzymes (20, 21). The peptide with Lys^4 mutated to arginine (peptide 9) was found to be a strong competitive inhibitor of LSD1 with a measured K_i of 0.4 μM. Among

the many peptides evaluated in this and previous studies (13, 22), peptide 9 exhibits the highest binding affinity and inhibitory power. To exclude that this tight binding simply reflects a particularly favorable interaction between the delocalized charge on the guanidinium group and LSD1 active site, we also probed guanidine, phenyl guanidine, and arginine for potential LSD1 inhibition. None of these compounds exerted any inhibitory effect on LSD1 activity indicating that, although an Arg side chain at position 4 significantly improved peptide binding, guanidinium or the isolated arginine amino acid was unable to bind to LSD1. In a perspective of exploiting chromatin as a therapeutic target, knowledge on mechanisms of LSD1 inhibition may help future drug design studies.

Oxygen Reactivity of LSD1—The LSD1-catalyzed demethylation reaction is an oxidative process that requires an electron acceptor to reoxidize FAD (Fig. 1). Although it has been shown that molecular oxygen can function as the electron acceptor substrate, other molecules were also shown to efficiently reoxidize the LSD1-bound flavin (6). This raises several questions. Can dioxygen function as physiological electron acceptor? Does the reduced enzyme react with oxygen when it is still bound to the demethylated histone? What is the order of the events during the catalytic cycle? To clarify these issues, we investigated the kinetics of reduced LSD1 with oxygen at pH 7.5 and 25 °C by mixing a solution of artificially reduced enzyme with buffer solutions containing various concentrations of oxygen in the stopped-flow instrument (Fig. 3). A plot of the pseudo first-order rate constant *versus* oxygen concentration was linear (data not shown) giving a bimolecular rate constant of 9.6×10^3 $M^{-1}s^{-1}$. This value is comparable with the rate constants typically found in other flavin-dependent oxidases (23) and indicates that LSD1 can be efficiently reoxidized by molecular oxygen. These observations suggest that *in vivo*, the enzyme likely acts as an oxidase using oxygen as the electron acceptor required for completion of the catalytic cycle. In addition, the measured rate constant value indicates that reoxidation by molecular oxygen is a relatively fast process compared with the turnover number measured in the steady-state kinetics experiments (13), implying that FAD reoxidation is not rate-limiting in the demethylation reaction. To dissect the order of events during the catalytic cycle, we analyzed the kinetics of the oxygen reaction in the presence of an unmodified histone H3 peptide known to be a LSD1 inhibitor (13) (peptide 10, Table 1). Addition of such peptide in stopped-flow measurements did not markedly influence the reoxidation rate (Fig. 3), suggesting that, after Lys^4 demethylation, FAD can be reoxidized by oxygen before release of the demethylated product, *i.e.* LSD1 is likely to function through a ternary complex mechanism (23). This observation has two important implications. First, in the case of a dimethylated H3-K4 substrate, the two methyl groups can be sequentially removed, whereas the protein stays bound to the histone substrate. Second, after completion of the oxidative demethylation reaction, LSD1 can remain firmly attached to the histone, functioning as a docking element for the LSD1-containing corepressor complexes.

In Vivo Assays of LSD1 Inhibition—To further investigate the ability of LSD1 to bind to a demethylated histone peptide, we performed an *in vivo* transduction experiment by delivering peptides into cells (Fig. 4, *a* and *b*). Two peptides were selected for this experiment: the demethylated peptide consisting of residues 1–21 (peptide 10), which competitively inhibited recombinant LSD1 (Table 1); and a shorter unmodified peptide corresponding to residues 5–21 (peptide 11), which only weakly inhibited LSD1 (K_i, 90 μM; Table 1) (13). We reasoned that the presence of an excess of each peptide in HEK293 cells should compete with the cellular histone H3 tails for the binding to LSD1. To test the effect of the selected peptides on endogenous LSD1, we monitored the expression of the *SCN1A* and *SCG10* genes that are targeted by LSD1-containing complexes (4, 24), compared with the expression levels of three different control genes, β_2-microglobulin (shown in Fig. 4*b*), β-actin, and β-tu-

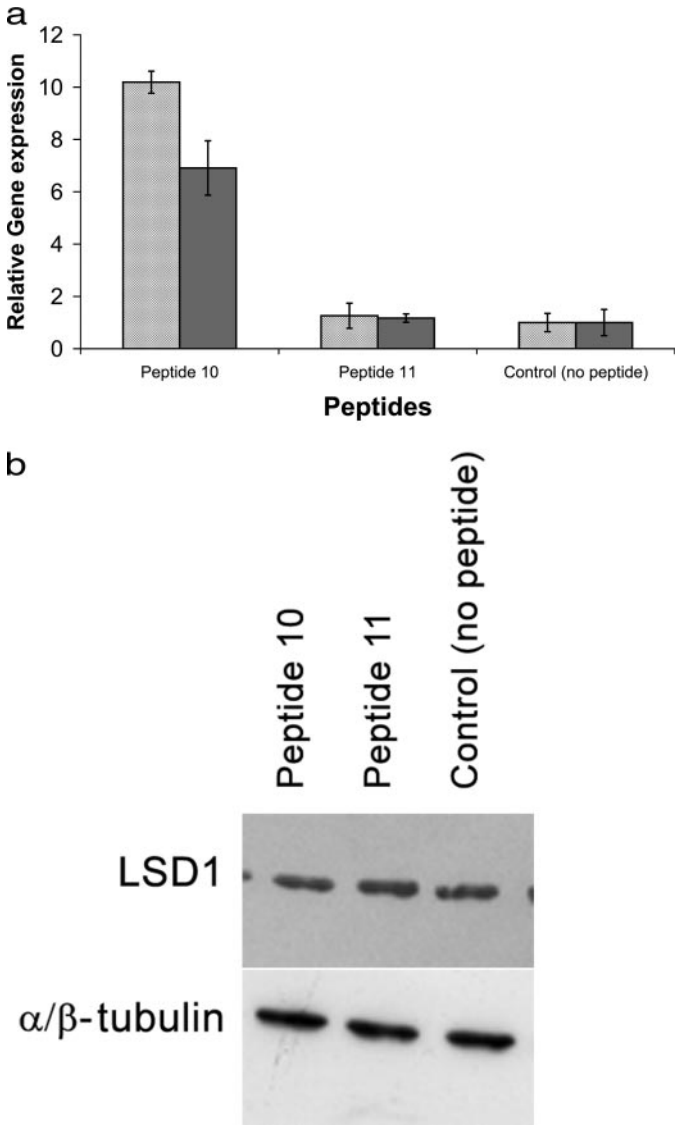

FIGURE 4. **Expression levels of LSD1 target genes.** *a*, change in the expression level of the LSD1-targeted genes *SCN1A* (*dotted bars*) and *SCG10* (*solid bars*) relatively to the housekeeping β_2-microglobulin gene expression. The expression level was monitored in the presence of different histone H3 N-terminal tail peptides: the unmodified peptide (*peptide 10*, Table 1), the shorter unmodified peptide (*peptide 11*, Table 1), or without peptide (*control*). *Error bars* represent standard deviation based on two independent experiments, each one analyzed in triplicate. *b*, Western blot of total proteins shows unchanged levels of LSD1 and α/β-tubulin in HEK293 cells treated with the above mentioned peptides or without peptide (control).

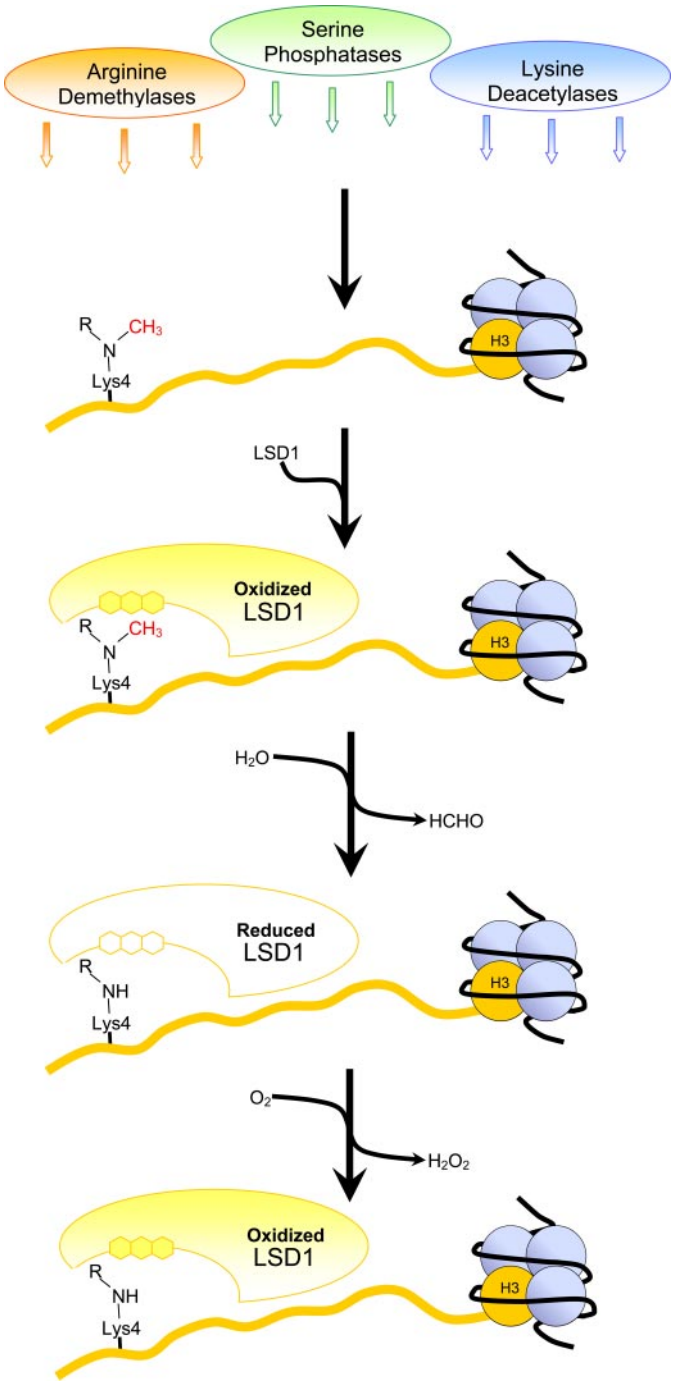

FIGURE 5. **Proposed model for the sequence of events leading to transcriptional repression.** Efficient demethylation of histone H3-K4 by LSD1 requires that the post-translational modifications on the same histone tail be removed. LSD1 is shown in either oxidized (*yellow*) or reduced (*white*) state. *R* indicates hydrogen or CH_3 in mono- or dimethylated Lys^4 of histone H3, respectively. LSD1 can complete its catalytic cycle remaining bound to the demethylated histone H3 tail.

bulin. We measured an increase in the expression of both *SCN1A* and *SCG10* in the presence of peptide 10 (Fig. 4, *a* and *b*), whereas cells treated with peptide 11 (a very weak inhibitor) displayed no variations in the expression of target genes. These data are consistent with the notion that H3-K4 methylation is an activation mark and provides a correlation between the *in vitro* inhibitory properties of the peptides and their ability of affecting the LSD1 activity *in vivo*.

DISCUSSION

Our results highlight the sophistication and specificity of histone H3 N-terminal tail recognition by LSD1. Within the recognized segment of 21 amino acids, essential for productive binding, we found that both post-translational modifications and conformational properties of the histone H3 tail are important factors in substrate recognition and Lys^4 demethylation. By using 21-amino acid peptides in biochemical assays, we previously demonstrated that phosphorylation on Ser^{10} drastically reduces LSD1 enzymatic activity (13). Here we show that hyperacetylation of Lys residues totally abolishes LSD1 function, whereas the strength of the effect of Arg methylation depends on the Arg residue that is modified, varying from a slight reduction in activity associated to Arg^{17} methylation to a complete loss of activity caused by methylation of Arg^8. Methylation of Lys^9 appears to be the only epigenetic modification that does not impair enzyme function (13). Taken together, these data indicate that epigenetic modifications on the H3 tail are first removed by other chromatin remodeling enzymes including histone deacetylases, arginine demethylases, and serine phosphatases that thereby "prepare" the histone tail for efficient LSD1-catalyzed Lys^4 demethylation (Fig. 5). The fact that LSD1 is typically found in association with histone deacetylases 1/2 is of particular interest in that it suggests that these two enzymes might function as a sort of "double-blade razor" that first eliminates the acetyl groups from acetylated Lys residues and then removes the methyl group from Lys^4, effectively pruning the histone H3 N-terminal tail.

The time-resolved stopped-flow experiments demonstrate that completion of LSD1 catalytic cycle through reoxidation of the FAD cofactor does not require release of the demethylated product. In addition, *in vivo* experiments showed that an unmodified 21-amino acid peptide (corresponding to the demethylated product) was able to reactivate target genes by inhibiting endogenous LSD1. On these bases we propose that after demethylating Lys^4, LSD1 can remain bound to the histone H3 tail (Fig. 5) possibly tethering other chromatin remodeling enzymes. In particular, LSD1-containing complexes can include enzymes that add methyl groups on Lys^9, a well known gene repression mark on Lys^4-demethylated histone (10, 25). In this view, LSD1 may trigger a process that leads to gene repression acting as a switch between chromatin states. We suggest that in specific contexts of genes targeted by LSD1-containing corepressor complexes, H3-K4 demethylation represents the removal of the last gene activation mark and that LSD1 recruits the forthcoming chromatin remodelers leading to the introduction of gene repression marks.

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